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(54) Title: OVINE ADIPOCYTE ANTIGENS AND THEIR USE IN THE IMMUNOLOGICAL CONTROL OF FAT

(57) Abstract

Antigens present in the plasma membrane of mature ovine white adipocytes, which are not detectable in ovine liver, kidney, spleen, brain, cardiac muscle, skeletal muscle or lung or in ovine erythryocytes, which react with antisera raised against said adipocytes and which on SDS-PAGE give rise to protein bands of relative molecular mass (r.m.m.) about 101 or about 21 Kilodaltons, respectively and antibodies thereto are useful for the reduction of fat in sheep.

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OVINE ADIPOCYTE ANTIGENS AND THEIR USE IN THE IMMUNOLOGICAL CONTROL OF FAT

Background of invention

1. Field of the invention

This invention relates to the immunological control of fat in the mammalian body, especially in non-human animals (herein referred to simply as animals).

2. Description of prior art

Excess fat in animals is recognised as detrimental to lowering production costs and a health risk to human consumers. One attempt at reducing the deposition of fat in animals has been to incorporate a β-agonist in the feed. β-Agonists have encountered many problems, particularly that they can adversely affect meat quality and its preservation during storage and that the animals have to be slaughtered within a short period after the compound is withdrawn from the feed. Another attempt has been the use of growth hormones such as bovine somatotrophin (BST). Besides stimulating milk yield, BST improves the protein : fat ratio and feed conversion efficiency in cattle. Although the dairy industry considers BST to be safe, it has been the subject of considerable concern to regulatory authorities and consumer groups.

In view of these problems, Flint et al. pioneered the idea of raising antibodies to the plasma membranes of adipocytes and injecting them into animals. It was found that this treatment reduced the amount of fat considerably and that this reduction was maintained for several months without inducing adverse effects. The first published reports, in which crude antisera raised against whole adipocyte plasma membranes from rats were shown to have such an effect, were by Flint, Coggrave, Futter, Gardner and Clarke, International Journal of Obesity 10, 69-77 (1986) and by Flint and Futter, Annual Report of the Hannah Research Institute, Ayr, Scotland 1986. Not only was fat reduced, but there was a body weight gain and an improvement in

feed conversion efficiency. In an article "Can obesity be controlled?" by Flint, Futter and Peaker, News in Physiological Sciences 2, 1-2 (February 1987) it is reported that similar antibodies have been produced against chicken, sheep and pig fat cells and that all are effective against adipocytes in vivo. A more detailed report on the effects of treatment of rats with anti-adipocyte antibodies is given by Panton, Futter, Kestin and Flint in American Journal of Physiology 258 (Endocrinol. Metab. 21): E985-E989 (1990). See also Moloney and Allen, Proc. Nutrition Society, July 1988 Meeting, page 14. Although Killefer and Hu, Proc. Soc. Exp. Biol. Med. 194, 172-176 (1990) have reported raising a monoclonal antibody to pig adipocyte plasma membranes, the hybridoma is believed not to be publicly available and the paper contains no evidence that the antibody would lyse fat cells.

Although the above work has demonstrated experimentally the possibility of treating fat deposition in vivo by the administration of anti-adipocyte antibodies, it is a problem that the production of such antibodies may be very labour-intensive. The administration of the plasma membranes themselves as antigens could be considered, if they could be conjugated to carrier proteins and could thereby be made "non-self". However, the production of plasma membrane material from slaughterhouses poses difficulty of quality control. If the antigen(s) responsible for the fat reduction could be isolated and purified, the way would be open to making them by a recombinant DNA method or by protein synthesis. However, preliminary work revealed that there are many common epitopes shared between adipocytes and other tissues and it was therefore doubtful whether highly adipocyte-specific antigens would be isolatable.

Shortly before the priority date of this patent application, a paper was published by Nassar and Hu, Comp. Biochem. Physiol. Vo. 98B No. 2/3, 361-367 (1991). These authors report ovine adipocyte-specific antigens of relative molecular mass 70, 106

and 110 KiloDaltons, but the term "specific" is inevitably qualified by the limited nature of the experiments conducted which depend on a comparison of Western blots of proteins separated on gels and then with only four other kinds of tissue. Nor is there any demonstration that their adipocyte antigens stimulate any reaction in sheep.

Summary of the invention

After considerable research, the inventors have isolated, from fat cell plasma membranes, antigens which appear to be specific to adipocytes (at least in the sense of not being detectable in many other body tissues of the animal) and reactive with antibodies to fat cell plasma membranes. However, not all of these antigens give rise to antisera which do, in fact, reduce fat cell deposition in vivo. After tests of rabbit anti-ovine fat cell plasma membranes in vivo on lambs, however, the inventors have found certain antigens which do appear to be able to produce such antibodies. Accordingly, the present invention provides two antigens present in the plasma membrane of mature ovine white adipocytes, which are not detectably present in ovine liver, kidney, spleen, brain, cardiac muscle, skeletal muscle or lung or in ovine erythryocytes, which react with antisera raised against said adipocytes and which on SDS-PAGE give rise to protein bands of relative molecular mass (r.m.m.) about 101 and about 21 KiloDaltons, respectively, as determined by markers of relative molecular mass 14,300, 21,500, 30,000, 46,000, 69,000, 92,500 and 200,000.

Eight candidate antigens, all potentially fat cell specific, were resolved by SDS-PAGE, with difficulty, but the only way in which it could be determined whether they would reduce fat was to raise antibodies and test them in sheep. As a result of these tests it was determined that Nos. 2 and 7, at least, are active.

The invention also provides the use of such antigens and antibodies thereto for the active or passive immunisation of sheep.

Brief description of the drawings

Fig. 1 shows a laser densitometry scan profile of ovine adipocyte plasma membrane polypeptides separated by SDS-PAGE;

Fig. 2 is a photograph of gels showing the protein bands obtained from various ovine tissues including adipocyte plasma membanes;

Fig. 3 is another laser densitometry scan profile of the ovine adipocyte plasma membrane polypeptides prepared on a larger scale and separated by SDS-PAGE, showing the positions of the 8 candidate antigens;

Fig. 4 is a photograph of a preparative gel showing the bands to be excised to obtain 8 candidate antigens; and

Figs. 5 and 6 are photographs of typical histological sections of back fat treated with control serum and antibodies of the invention respectively.

Description of the preferred embodiments

The adipocyte membranes used to prepare the antigens can be obtained from any ovine breed as it is likely that they will be highly conserved between breed. It is suggested that they be obtained from mature white adipocytes. A mature adipocyte is one which demonstates morphologically and biochemically the adipocyte phenotype including the presence within the cell of a large central unilocular lipid droplet. All references to "adipocytes" herein mean mature adipocytes. It is also suggested that the adipocytes be obtained by a gentle procedure which does not damage them and which removes extracellular material. It has been found that a Clostridium histolyticum collagenase enzyme is particularly useful for such removal. The cells are then allowed to recover by a conditioning step of incubation at a temperature of 38-42°C for a period of several hours, e.g. 2 to 24 hours.

The adipocytes can then be separated and washed by flotation, retaining the floating adipocyte layer, which is then homogenised and the plasma membranes separated on a Percoll gradient in which they appear near the top of the gradient

tube. "Percoll" is a colloidal polyvinylpyrrolidone-coated silica and is a registered trade mark of Pharmacia. It is then necessary either to remove non adipocyte-specific antigens or to identify which of the antigens present in the extract are adipocyte specific. For this purpose, other ovine tissue is required. The greater the number of kinds of tissue employed for this purpose, the better the chances will be of obtaining adipocyte-specific antigens. While it is possible to raise antisera to antigens from other tissue and then absorb the whole adipocyte plasma membrane (WAPM) extract on these antisera, the present inventors have found it preferable to make a direct protein band immunoblot comparison by running plasma membrane extracts prepared from various ovine tissues on SDS-PAGE, Western blotting and then immunoblotting specific protein bands with antibodies raised against plasma membranes of adipocytes. This immunodetection was carried out with difficulty, by scanning laser densitometry. Comparing the laser scans from the adipocyte plasma membranes with those from other tissues, eight candidate "adipocyte specific antigens" were identified from the blots of a large number of proteins.

In order to generate sufficient material by this method, large scale preparations were carried out on SDS-PAGE and the bands containing each of the eight antigens were cut from the gels and proteins were then electroeluted therefrom. Antisera were raised against the 8 electroeluted products and 7 of these were found to react immunogenically with whole adipocyte plasma membranes. The antisera were then tested <u>in vivo</u> by passive immunisation of lambs by local injections into areas of the body having significant subcutaneous fat. The largest lesions, indicating the reduction of fat, resulting from this treatment were those induced by anti-WAPM, anti-antigen 2 and anti-antigen 7, although some useful result was obtained from antigens, 1, 3 and 4. The invention therefore includes these antigens, especially No. 3, independently of antigens 2 and 7.

Some preliminary amino acid sequencing of antigen 7, the most promising of those tested, gave a partial sequence (SEQ ID NO: 1):

Val Xaa Pro Arg Xaa Xaa Ile
1 5

proceeding left to right in an N-terminal to C-terminal direction, wherein Xaa are unknown amino acids. That present in position 2 is probably Ile and that present in position 5 is Leu or Arg. (The third unknown amino acid might be the same as or different from the other two. Sequence listing requirements of the US Patent Office compel us to use the same symbol "Xaa" throughout, even though the amino acids might be different). It is not known whether this is a sequence of the whole, mature protein or only of a smaller electroeluted fragment thereof. If the latter, it might not be an N-terminal sequence.

The information given herein enables antigens to be identified, isolated and, by methods well known in the art, purified. It is then possible to prepare antibodies by conventional raising of antisera or production of monoclonal antibodies by the Köhler-Milstein method and variations thereon. Immunogenic portions of natural antibodies are also included within the scope of the term "antibodies" as used herein, as are also hybrid human-mouse antibodies and other antibody-like products obtainable by recombinant DNA methods.

The antibodies thereby produced will be used to help identify antigens from the adipocyte membrane by a recombinant DNA method. The preparation of such antigens was begun by producing a cDNA library from RNA obtained from isolated adipocytes using a guanidinium isothiocyanate extraction method (Chomczynski and Sacchi, Analyt. Biochem. 162, 156-159 [1987]) followed by affinity chromatography to enrich for poly A^+ RNA (Avid and Leder, Proc. Natl. Acad. Sci. (USA) 69, 1408-1412 [1972]). The cDNA prepared from this enriched RNA was inserted into the phage expression vector λgt 11. The correct clones will be identified by using either the antibodies previously

mentioned (Mienendorf et al. Methods in Enzymology 152, 451-457 [1987]) or by synthesising a mixture of the most probably complementary oligonucleotides as determined from amino acid sequence of the antigens and considerations of normal DNA codon usage, as probes. The clones thus identified and isolated, after confirming their binding to antibody, can be used to express the protein of interest.

The main use of the invention will be for the treatment of fat in sheep. While either active or passive immunisation is likely to have an effect, active immunisation is preferred and for this purpose the antigen will have to be made "non-self" so that it does not suffer host immune tolerance. This can be achieved by any of the conventionally explored methods, especially by conjugation to a carrier protein such as rabbit serum albumin or KLH. It is also probable that epitopes of the antigen will be identifiable in due course, possibly enabling shorter-chain peptides to be used as immunogens. The invention includes these within its scope. Such peptides can also be used in the form of conjugates or other elaborated structures such as branched forms thereof.

The favoured proposed route of administration for active immunisation is by subcutaneous injection. Amounts of antigen in the range of 1 µg to 1g. per treatment are envisaged, the animal being treated preferably once only for reasons of convenience of husbandry. Adjuvants such as small components of Freund's Complete Adjuvant or peptides can be employed. Oral or nasal routes are possible alternatives.

For passive administration, the antibodies are preferably given without an adjuvant, again preferably by subcutaneous injection. Other routes such as intraperitoneal are possible.

The following Examples illustrate the invention.

EXAMPLE

Section 1

Isolation of mature white adipocytes from ovine adipose tissue.

Methods

Adipose tissue was collected from the following depots of freshly killed Welsh Mountain x Dorset Horn Sheep : channel, mesenteric, perirenal, subcutaneous and intermuscular. The mixed adipose sample was rinsed and then transferred to Krebs Ringer Bicarbonate (KRB) buffer (120.5mM NaCl, 13.1mM $NaHCO_3$, 2.6mM KCl, 1.2mM KH_2PO_4, 1.2mM $MgSO4.7H_2O$, 1.2mM $CaCl_2.6H_2O$) containing penicillin (100mg/l), streptomycin (100mg/l) and Fungizone (2.5mg/ml) which was held at 42°C.

Adipocytes were prepared from this tissue by the method of Plaas and Cryer, J. Develop. Physiol. 2, 275-289 (1980) as previously applied to sheep by Cryer & Cryer, Biochem. Soc. Trans. 17, 1120-1121 (1989). During the isolation, all solutions and apparatus were maintained at a temperature of 42°C.

Following the removal of the visible blood vessels by dissection, the adipose tissue was rinsed in KRB buffer and transferred to a solution of bovine serum albumin (4% w/v) in KRB, containing 1mg/ml Clostridium histolyticum collagenase (type II, specific activity 590 units/mg dry weight). The tissue was then chopped finely with scissors and incubated in a siliconized glass vessel at 42°C with constant stirring for 60 mins.

After the 60 min. incubation period, the digested material was filtered through a nylon mesh (250µm diameter) into plastic centrifuge tubes. The resulting suspension was then centrifuged at 200g.av. for 15 seconds. This period of centrifugation resulted in the adipocytes forming a loose plug over the liquid infranatant. The infranatant was then removed using a plastic Pasteur pipette, and the adipocytes that remained washed free of residual collagenase by resuspension in KRB buffer 3 times with centrifugation at 380g for 5 min. and removal of the infranatant

each time. After washing, the cells were resuspended in medium 199 and transferred to 25ml tissue culture flasks. The flasks were then incubated at 42°C for 4 hours. Such a "conditioning" step maximises immunoreactivity versus anti-adipocyte serum.

Following this incubation, the resulting "conditioned" cells were then used immediately or frozen in liquid nitrogen before being stored at -70°C.

The cells isolated in this manner were shown to be greater than 80% intact and viable using standard cell viability tests, and maximal immunoreactivity had been obtained.

Section 2

The conditioning of ovine adipocytes following isolation in order to ensure full recovery of surface immunoreactivity

It has been demonstrated previously that cells isolated by enzymatic means can suffer considerable damage during their preparation. Attempts by other workers to produce isolated adipocytes non-enzymatically did not remove all contaminating extracellular material and produced a poor yield of cells which were heavily contaminated with lipid droplets. Such contaminating extracellular material co-purifies with plasma membranes isolated from whole tissue. Previous studies relating cell surface immunoreactivity to the condition of cells following their enzymatic isolation, Al-Jafari, Lee, Tume and Cryer, Cell Biochem. Function 4, 169-179, (1986), indicated that the use of collagenase concentrations, predetermined as the minimum required for tissue digestion (and which varies with batch), together with a post-isolation period of incubation of 4h in nutrient medium, lead to the expression of maximal levels of surface immunoreactivity. Thus this procedure was adopted to ensure that the plasma membranes isolated from such cells would contain the fullest range and pattern of membrane surface components, making them, as close as possible, equivalent to the state characteristic of the adipocyte in vivo.

Section 3

The isolation and preliminary characterization of plasma membrane preparations from conditioned ovine adipocytes

Methods

Adipocytes, isolated as described in Section 1 and conditioned as described in Section 2, were used for the preparation of plasma membranes. The method used was essentially that described by Belsham, Denton and Tanner, Biochem. J. 192, 457-467, (1980), with slight modifications as described by Cryer, Gray and Woodhead, J. Develop. Physiol. 6, 159-176, (1984). Typically, 30ml of adipocytes were mixed with 30ml of sucrose-based extraction medium (0.25M sucrose, 10mM Tris-HCl, pH 7.4, 2mM EDTA) prewarmed to 42°C. The suspended cells were then disrupted by mixing for 3 x 30 sec on a vortex mixer. The final homogenate was centrifuged at 1000g.av. for 30 seconds. Following centrifugation, the resulting infranatant was removed from beneath the floating plug of fat by aspiration and kept on ice. The floating fat was then re-extracted with a further 20ml of extraction medium at 42°C, as described previously, and the centrifugation repeated. The second infranatant was then pooled with the first and this combined defatted homogenate was centrifuged at 30,000g for 30 min at 4°C. The supernatant was then removed and the pellet resuspended in 500µl of the extraction medium using a plastic Pasteur pipette. Density gradient centrifugation was then used to separate mitochondria and other components. For this, Percoll was mixed with gradient medium (2.0M sucrose, 80mM Tris-HCl, pH 7.4, 8mM EDTA) and the sucrose-based extraction medium in the ratio 7:1:32 (by volume). The resuspended particulate fraction (500µl) was then dispersed into the Percoll gradient mixture (8ml) and the mixture centrifuged at 10,000g for 15 min. During centrifugation, the Percoll formed a density gradient through the solution from 1.035g/ml at the top of 1.08g/ml at the bottom. After centrifugation, two clearly defined bands were present, one just below the surface at the

top of the tube (plasma membrane) and one close to or at the bottom of the tube (crude mixed mitochondrial fraction). The putative plasma membrane band was removed by aspiration using a plastic Pasteur pipette.

To remove Percoll and sucrose from the membranes, they were washed by mixing with 35ml of washing medium (0.15M NaCl, 10mM Tris-HCl, pH 7.4) followed by re-centrifugation at 15,000g for 15 min. The resulting pellet was washed again using the same procedure. Finally the washed, pelleted membranes were resuspended in 0.5ml of the sucrose-based extraction medium and stored at -20°C. The recovery of plasma membrane fractions were adequately pure, as determined by assaying 5'- nucleotidase activity, Newby, Luzio and Hales, Biochem. J. 146, 625-633, (1975), and by the determination of protein.

Section 4

The replicated SDS-PAGE analysis of the pattern of polypeptides from ovine adipocyte plasma membranes.

Methods

Preparation and running of analytical gels

The above-prepared plasma membranes from ovine adipocytes were analysed by SDS-PAGE using a linear gradient of polyacrylamide concentration between 8 and 20% for the resolving gel. The gels were prepared and run by a modification of the procedure originally outlined by Laemmli, Nature, 227, 680-685, (1970) in a vertical electrophoresis unit (Atto Corporation) according to the manufacturer's instructions. The electrophoresis was performed in 7 by 8cm. "minigels" (0.75mm. thick). See also Tume, Lee and Cryer, Comp. Biochem. Physiol. 80B, 127-134, (1985).

The acrylamide concentration gradient was created using a gradient maker composed of two 20ml. chambers. The 8% and 20% resolving gels [2.5ml stock acrylamide solution consisting of 40% (w/v) acrylamide and 1% (w/v) N,N'-methylenebisacrylamide in H_2O (for the 20% gel) or 1.0ml stock acrylamide solution and 1.5ml distilled water (for the 8% gel), 2.5ml resolving gel

buffer, 0.375M Tris-HCl, pH 8.8, containing 0.1% (w/v) SDS, 30µl ammonium persulphate (75mg/ml in H_2O), 20µl TEMED (1% v/v in H_2O)] were added to the respective chambers. The acrylamide solution was pumped from the gradient maker at a flow rate of 4ml/min using a peristaltic pump, to the lower edge of the glass plate sandwich. When the acrylamide gradient addition was complete, the unpolymerised solution was overlayed with butan-1-ol (0.25ml.). After polymerisation, (approx. 1h), the butan-1-ol layer was removed and the gel surface washed with distilled water and then blotted dry. A well-former was inserted, and 5% (w/v) acrylamide stacking gel mixture [0.625ml stock acrylamide solution (as above), 2.5ml stacking gel buffer (as above), 1.725ml distilled water, 13µl TEMED (1% v/v in H_2O), 50µl ammonium persulphate (75 mg/ml in H_2O)] poured into the remaining space in the plates to form sample wells.

Plasma membrane preparations and protein standards (Amersham "Rainbow" markers; r.m.m. 200,000, 92,500, 69,000, 46,000, 30,000 21,500 and 14,300) were heated to 100°C for 5 minutes in the presence of an equal volume of loading buffer. [1.0ml stacking gel buffer, 0.125M Tris-HCl, pH 6.8, containing 0.1% (w/v) SDS, 80mg SDS, 400µl 10% glycerol, 200µl 2-mercaptoethanol, 100µl 0.25% bromophenol blue, 300µl distilled water]. Electrophoresis at 40mA constant current was carried out according to the manufacturer's recommendations. The electrode buffer used was 0.25 M Tris containing 0.192 M Glycine and 0.1% (w/v) SDS, at pH 8.3.

Staining and destaining of gels

Gels were immersed for 1-2 hours in 0.125% (w/v) Coomassie Brilliant Blue in 5% (v/v) glacial acetic acid containing 50% methanol. To visualise protein bands, the gels were then destained in repeated changes of 7% (v/v) acetic acid in 10% (v/v) methanol.

Calibration using standards of known relative molecular mass

The relationship between relative molecular mass and mobility was determined by loading analytical gels with a

standard mixture of prestained polypeptides of known relative molecular mass (Amersham "Rainbow" Markers), as recited in the "Summary of the invention". The relative mobility of each polypeptide was calculated by manual measurement of the gels, plotted against $\log_{10}$ molecular mass. The gradient, calculated by regression analysis, was found to be linear.

Laser scanning densitometry

The gels, stained and de-stained to reveal protein bands, were scanned by a laser scanning densitometer, whereby the intensity of the bands is converted into a peak height and their size is represented by the area under the peak. Table 1 shows the location of the bands, the relative molecular mass of the proteins determined from the calibration plot and the relative amounts of protein represented by areas under peaks. In Fig. 1 of the drawings, the SDS-PAGE bands are matched to the laser densitometric scan. Table 1 is labelled to indicate 7 bands which were later identified to contain proteins specific to adipocyte plasma membrane components. (Antigen 7 has the characteristic of not staining well with Coomassie Blue in analytical gels. However, this is less of a problem n preparative gels and the identification of this antigen by its immunoreactivity does not depend on protein staining).

Table 1

Peak No.	Retention Factor	Calculated molecular mass	% relative area	Specific adiopcyte plasma membrane components (see Section 7/8)
1	.9615	14246	8.0	Antigen 8 14.8-17.5K
2	.9457	14927	1.3	
3	.9287	15700	3.5	
4	.9162	16289	6.5	
5	.9042	16881	8.4	
6	.7525	26472	1.6	Antigen 6 26/27K
7	.7328	28028	2.2	
8	.7109	28556	2.3	
9	.6868	32114	2.3	
10	.6687	33876	2.4	
11	.6325	37713	5.0	
12	.5849	43411	1.5	
13	.5517	47896	6.1	
14	.5245	51903	4.5	
15	.4981	56130	1.2	
16	.4830	58694	7.4	Antigen 5 55.2K
17	.4287	68912	1.6	
18	.4060	73705	6.8	
19	.3917	76895	3.5	
20	.3683	82413	5.6	Antigen 4 82K
21	.3306	92129	1.1	
22	.3079	98537	7.0	Antigen 3 92K
23	.2709	109925	1.6	Antigen 2 100-104K
24	.2453	118576	1.6	
25	.1977	136489	1.5	Antigen 1 145K
26	.1373	163230	3.1	
27	.1170	173300	2.5	

5. Comparison of (1) the pattern of reactive bands seen with adipocyte plasma membranes with (2) similarly treated membranes prepared from other ovine tissues; and;

6. The use of such comparisons to identify potentially adipocyte specific antigens of the adipocyte membrane.

Methods

Preparation of ovine plasma membranes from tissues other than adipose.

Collection of tissues

Tissues, other than adipose, were excised from freshly killed Welsh Mountain X Dorset Horn Sheep. The samples were cut

into small pieces (2g approx.) and immersed in liquid nitrogen until completely frozen. The material was then stored at -70°C until used.

Blood, for the preparation of red blood cell (rbc) membranes, was collected from the jugular vein of live sheep into heparinised tubes.

Preparation of tissue plasma membranes

15g of each tissue was placed in a plastic centrifuge tube together with 15ml of Membrane extraction Medium (MEM) consisting of 1.4g Na_2HPO4, 81.5g sucrose, 0.83g EDTA per litre and 15µl of phenylmethylsulfonyl fluoride solution (3.48mg. ml^{-1}, 20mM). This sample was then homogenised using a tissue homogeniser (3 x 10 second bursts), and the resulting homogenate centrifuged at 20,000g for 20 mins to remove particulate matter. The resulting supernatant was then centrifuged at 100,000g for 1 hour in a Beckman (L8-70M) ultracentrifuge. The resultant pellet was resuspended in 40% sucrose in MEM.

Sucrose gradients were prepared in polypropylene tubes by overlaying solutions of 0, 32 and 36% sucrose. The pellet, suspended in 40% sucrose in MEM was then applied to the bottom of the gradient using a peristaltic pump. The prepared gradient was then spun at 100,000g for 1 hour.

Following centrifugation, a diffuse band of material was visible at the 32/36% interface. This was removed carefully by aspiration and washed in MEM without sucrose. Finally, this membrane-containing fraction was resuspended in PBS.

Preparation of ovine red blood cell ghosts

Ovine red blood cell ghosts (i.e. de-haemoglobinised) were prepared using the method of Raval and Allan, Biochem. Biophys. Acta. 856, 595-601 (1986). For this, 60ml of fresh blood was collected into 10ml heparinised tubes and spun at 2010 g. av. for 10 min. The plasma was then removed by aspiration and the remaining packed red cells washed three times by addition of 10ml of isotonic Tris/HCl buffer (5.0mM Tris, 150mM NaCl, 4.2mM HCl, adjusted with 1M NaOH to pH 7.4) followed by centrifugation

at 2010 g. av. for 10 min. The cells were then lysed by the addition of 10ml of hypotonic Tris/HCl buffer (5.0mM Tris, 4.2mM HCl, adjusted with 1M NaOH to pH 7.4). The cells suspended in this solution were left at room temperature for 10 min. and then the mixture was centrifuged at 38720 g. av. for 10 min. The sedimented material was washed 3 times in the lysis buffer. Following this procedure, the membranes, when collected as a pellet, were pale pink to white in colour, with little residual haemoglobin present.

SDS-PAGE analysis

Fig. 2 shows the pattern of bands stained for protein when several of the ovine tissue plasma membranes were analysed using SDS-PAGE.

Key to Figure 2:

1, 5, 9, 12 & 15 = "Rainbow" (Amersham) relative molecular mass standards

2 & 3 = liver plasma membranes

4 = adipocyte plasma membranes

6 = brain plasma membranes

7 = spleen plasma membranes

8 = kidney plasma membranes

10 = red blood cell plasma membranes

11 = skeletal muscle plasma membranes

13 = "Electran" (BDH) relative molecular mass standards

14 = lung plasma membranes

16 = cardiac muscle plasma membranes

The gels in tracks 1 to 10 were stained with Coomassie Brilliant Blue, and those in tracks 11 to 16 by silver staining.

Laser densitometry of SDS-PAGE gels and immunoblots

First, (a) blots of SDS-PAGE gels (as above) that had been stained with Coomassie brilliant blue and (b) immunoblots of the same gels against equine anti-ovine adipocyte plasma membranes, stained with 4-chloro-1-naphthol, were scanned using an enhanced laser densitometer (Ultroscan XL LKB). Data collected by the laser densitometer was analysed and stored using the Gelscan XL

software package. This allowed comparisons to be made of the polypeptide composition of membranes resolved on SDS-PAGE and of immunoblots of membrane polypeptides (see below).

The relative molecular masses of specific proteins were determined by comparison of the stained bands with scans of the "Rainbow" standard relative molecular mass markers, as described below. For interest, the "Electran" markers in track 13 had the following relative molecular masses: Cytochrome c (equine) 12,300; Myoglobin (equine) 17,200; Carbonic anhydrase (bovine) 30,000; Ovalbumin (hen egg) 45,000; Albumin (bovine serum) 66,250; Ovotransferrin (hen egg) 76-78,000.

Equine anti-ovine adipocyte plasma membrane was found to interact with plasma membranes prepared from other ovine tissues, especially the liver. Therefore, laser densitometric scanning, of the reactive bands in adipocyte plasma membranes and in membranes from other tissues, was used to identify adipocyte- specific antigens. That is, immunoblots from the gels of whole adipocyte plasma membranes were compared with immunoblots from the gels of membranes from other tissues, viz. ovine liver, kidney, spleen, brain, cardiac muscle, skeletal muscle and lung and from gels of preparations of erythrocytes (red blood ghosts).

Since the major cross-reactive membranes are of liver cells, the equine anti-ovine adipocyte plasma membrane serum used for immunoblotting was pre-absorbed with whole liver tissue. In this way, it was hoped to make the equine serum specific to antigens of adipocyte membranes which are not present in the liver.

Materials and Methods

1. Preparation of gels and blots for scanning.

Each blot was trimmed so that the top of the blot marked the start of the original gel and the bottom marked the dye front. Since the "Immobilon" membrane has a high refractive index, the exact positions of the top and bottom of the blot were very clear on the scan. This was crucial for the accurate

calculation of the distances run by the dye front (Df) and by the proteins (d). Amido black-stained molecular weight markers from the same blot were treated similarly and laid on the densitometer next to their relevant blot.

2. Using the Ultroscan XL enhanced laser densitometer.

One-dimensional analysis was selected and a line beam with RS-232 output. In the "plot" menu, no smoothing was selected, so that all peaks were shown on the scan, and a "normalised" A-axis was used (i.e. the highest peak determined the absorbance scale on the Y-axis). The "delta binary" data format gave the most rapid transfer of information from the densitometer to the computer. Finally, the laser was set to x-width = 2, i.e. total width of each scan = 1.6mm, and the position and length of each track to be scanned was entered. The ruler was moved until the black line was over the centre of the track or the area of clearest band definition.

3. Use of the Gel Scan XL Laser Denitometer Software.

The integration function displays the scan on the screen and finds the position, height and area of each peak. The default integration parameters of peak width for peak search (3mm) and peak area rejected (0.02 AU.mm) were used. The "signal" method of integration was used and a "common" horizontal baseline, in which the computer disregarded the lowest 5% of data values then averaged the next lowest 5%, was drawn.

The positions of the start and end of the blot were noted and the distance run by the dye front (Df) was calculated. Then the part of the scan showing all the protein peaks was printed, together with a table showing the peak locations. The distances run by the protein peaks were calculated (the location of the start of the blot subtracted from the location of each protein) and, from these values, the relative mobility (R = d/Df) of each protein was determined.

4. Identifying adipocyte-specific immunoreactive plasma proteins.

The patterns/scans of reactive proteins from adipocyte plasma membranes were different from the patterns of the membrane proteins from other ovine tissues and which were themselves different from each other. However, closer comparison showed that many of the reactive proteins had similar mobilities although some appeared at specific different intensities of staining, related to the membrane type in question. To identify accurately those proteins that were definitely present only in adipocyte membranes on the blot, the Rf values of all the detected proteins in the different tissues were compared with the Rf values of the proteins from adipocytes. If any of the other tissues on the blot had proteins within 0.003 Rf units of any adipocyte membrane protein, then that protein was assumed to be not specific to adipocyte membranes.

This Rf comparison was performed for each blot separately, then the Rf values of amido black stained molecular weight markers from each blot were used to plot calibration curves of molecular weight against Rf on logarithmic paper. These calibration curves were used to find the molecular weights of these initially identified adipocyte specific immunoreactive membrane components. The relative molecular masses of the specific adipocyte membrane proteins determined using separate blots were listed and compared. Those identified on four or more blots were considered to be reproducibly detectable adipocyte specific immunoreactive membrane components worthy of further investigation.

Results

Eight adipocyte-specific antigens were thus found, having the relative molecular masses shown in Table 2 below. The number of determinations made was between 4 and 7 for each antigen. The "Rainbow" markers were used.

Table 2

Antigen number	Mean Relative Molecular Mass
1	143 ± 2.4
2	101 ± 0.8
3	91.5 ± 3.3
4	80 ± 1.6
5	52.8 ± 3.9
6	25.7 ± 1.4
7	20.9 ± 1.1
8	15.7 ± 0.7

Section 7

Preparation of the above-identified antigens on a larger scale

14 x 12 cm gels (1.5 mm thick) were used to resolve larger samples of ovine adipocyte plasma membranes. The buffers and gel solutions used were as for the analytical gels, but scaled up appropriately in volume, i.e. 5 litres of electrode buffer; 3 x resolving gel volume; 2 x stacking gel volume. The gels were prepared and run in the same way, but in a larger electrophoresis unit. They were stained with Coomassie blue as described in Section 4. The procedure was found to be reproducible.

The protein bands were found to occur reproducibly in the same positions on the gels in this larger scale preparation. Representative laser densitometric scans of tracks from such preparative gels, with the bands equivalent to the previously identified adipocyte specific plasma membrane antigens are shown in Fig. 3. Using the integration function in the scanner software, the relative proportions of the adipocyte-specific antigens (1 to 8) were estimated and are shown in Table 3 below.

Table 3

Antigen No.	Relative Molecular Mass	Rough estimate of % of total protein stain absorbed
1	143	2.5
2	101	1.0
3	92	4.0
4	80	0.9
5	53	1.1
6	26	1.8
7	21	2.6
8	16	1.4

The bands (33, 31, 30, 28, 25, 9, 7, 3) from which polypeptides (1-8) were to be eluted were excised from the gels at the positions shown in the gel photograph of Fig. 4 and placed in the sample wells of an electrophoretic concentrator ("Electrophor" Pharmacia). The proteins were electroeluted in a buffer of 50mM Tris, 50mM glycine, 0.1% SDS, into a highly conductive salt barrier which is provided in a constriction in a solution bridge between the two electrodes. At this barrier the electrophoretically eluted molecules become concentrated. The barrier was provided by use of a buffer which is the electroelution buffer but with 1M NaCl added. A potential difference of 100 volts was then applied for 60 min. or until the Coomassie blue stain had completely left the gel slices. The concentrated, eluted samples were removed from the instrument by microsyringe.

Section 8

Removal of SDS and renaturation of polypeptides by dialysis against QAE Sephadex and non-ionic detergent.

Removal of the high salt concentration present in the protein samples after elution and exchange of SDS for a non-ionic non-denaturing detergent "Nonidet P40" was achieved by dialysis using a modification of the procedure of Hjertan, Biochem. Biophys. Acta. 736, 130-136 (1983).

After elution as described above, the samples were placed in 6.3mm. dialysis tubing that had been boiled twice for 10 min. in water containing 1mM EDTA. They were then dialysed against 5 litres of 0.2mM "Nonidet P40", containing 2g of the anion exchanger QAE Sephadex, to improve the efficiency of SDS removal. Dialysis was carried out for 25 hours at room temperature with the dialysate being changed after 12 hours.

Following dialysis, the samples were freeze-dried and stored at -20°C.

Freeze-dried samples of the dialysed materials containing antigens 1, 2, 3 and 4 respectively were boiled in loading buffer (as described in section 4), run down SDS/PAGE gels and the gels stained for protein with Coomassie Blue solution. The original pattern of staining revealed that cleavage of the original polypeptides had occurred.

Section 9

The use of the individual electroeluted membrane components as immunogens.

For primary immunizations, glutaraldehyde was used to conjugate individual membrane antigens to a carrier protein, ovalbumin (Sigma, crude 53,000) in order to improve immunogenicity. The method used for the conjugation was a modification of that described by Reichlin et al., Proc. Soc. Exp. Biol. Med. 128, 347-350 (1968). Thus, for example, 25ug of adipocyte plasma membrane protein, Band 1, that had been electroeluted, renatured and freeze dried as described in section 10 was rehydrated in 100µl of 0.1M sodium phosphate buffer (pH 7.4). A solution of ovalbumin in 0.1M sodium phosphate buffer (100µl) was then added to give a 1:1 molar ratio of membrane protein:ovalbumin. 100µl of glutaraldehyde solution (in 0.1M phosphate) was then added, slowly and with vortex mixing, to give a protein: glutaraldehyde ratio of 1:11.

After 4 hours at room temperature the reaction was stopped by the addition of L-lysine (0.01M) to give a molar excess over that of glutaraldehyde and the mixture was left at room

temperature for 1 hour.

This procedure was repeated for the other 7 adipocyte specific antigen proteins, using similar amounts of protein.

300µl of each antigen conjugate, produced as described above, was emulsified in 400µl of Freund's Complete Adjuvant, and a stable emulsion prepared by vigorous vortex mixing. The emulsions were then administered to individual rabbits (New Zealand white strain) by multiple site, intradermal injection (Vaitukitus et al., 1971). Six weeks later, a secondary injection was prepared and administered in the same way, using the same amount of conjugated protein, but in this case emulsified in Freund's Incomplete Adjuvant.

Serum was obtained from each rabbit by removing blood from the ear vein, 7 days after the secondary injection. The blood was allowed to clot at room temperature for 2 hours and then left at 4°C overnight for the clot to retract. Finally the mixture was centrifuged at 2500g. av. for 15 min., the serum removed by aspiration and dispensed into 1ml aliquots and stored at -20°C.

A tertiary boost was administered at approx. 15 weeks. This tertiary boost consisted of approx. 25ug of freeze dried protein antigen (1 to 8) (unconjugated) rehydrated in 300µl PBS and emulsified in 300µl of Incomplete Freund's Adjuvant. The method and site of immunisation was as for the primary and secondary injections. A second serum sample was obtained from the rabbit by bleeding from the ear vein, 10 days after the tertiary boost. This sample was treated as above and stored at -20°C until use.

Section 11

The demonstration of antibody response when each of the 8 individual antigens were administered as immunogens using:

: ELISA

: Dot Blotting

: Immunoblotting

Serum samples collected from rabbits injected with each antigen (1-8) as in Section 9 were tested for their immunoreactivity with adipocyte plasma membrane (ELISA, Dot-blotting), electrophoresed samples of individual antigens or whole adipocyte plasma membranes electroblotted onto Immobilon.

ELISA

Ovine adipocyte plasma membranes (1ug/well) were coated to 96 Well plates and goat anti-Rabbit IgG conjugated with horseradish peroxidase was used to detect antibody binding from the antisera raised in rabbits against electroeluted antigen samples. Samples arising from rabbits after the secondary (1st bleed) and tertiary injections of immunogen (2nd bleed) were tested against ovine adipocyte plasma membranes and against plates coated with ovalbumin (1µg/well). Antiserum collected from a rabbit previously treated with ovine whole adipocyte plasma membranes was used as a positive control. The antisera, with the exception of that raised against whole ovine adipocyte plasma membranes, were also tested for reactivity using ovalbumin-coated to the plates. Although the antisera raised against whole ovine adipocyte plasma membranes reacted strongly with the membranes immobilized on the plate, none of the antisera against specific antigens (1st bleed) did so. However, after the rabbits had received a further boost of specific antigen the antisera (2nd bleed) in some cases showed positive reactivity in the ELISA. Thus, Antigen 1 sera showed a very slight specific reactivity, Antigen 2 sera showed a strong reactivity (stronger indeed than the antisera to whole ovine adipocyte plasma membranes at the same dilution) and much enhanced over that seen with the 1st bleed; Antigen 3 sera

showed a very slight specific reactivity as did Antigen 4, 6 and 7, with Antigen 5 sera being completely negative.

Dot blotting

The immunoreactivity of anti-ovine whole adipocyte plasma membrane (anti-WAPM) serum and antisera to some of the eight individual antigens was tested using a method of immuno-dot blotting, Heath et al., Biochem. Soc. Trans. 13, 1162-1163 (1985). Individual antigens were cut from a Coomassie blue-stained preparative gel (see above).

The immuno-dot blot procedures were performed at room temperature. Whole adipocyte plasma membranes (WAPM) (125, 62.5 and 31.3 ng) in 5µl of Tris-buffered saline (Tris 6.25g./l., NaCl 8.5g./l., adjusted to pH 7.5 with HCl) were applied to to separate strips of nitrocellulose paper. Further protein binding to the nitrocellulose was blocked by the incubation of the strips in Tris Buffered Saline containing 0.5% (v/v) "Tween-20" for 1h at room temperature or 4°C overnight. The strips were then incubated with antisera (anti-WAPM, anti-antigen and non-reactive sera at 1/750 dilution) for 2h, washed thoroughly with TBS, then immersed in goat anti-rabbit IgG diluted 1/1000 for 1h. After another thorough wash, the strips were immersed in rabbit peroxidase anti-peroxidase. This is a complex used in immunohistochemical detection procedures, see e.g. Al-Hussami et al., J. Immunol. Methods 113, 61-73 (1988) and Ogata et al., ibid. 65, 75-82 (1983), which is more sensitive than the conventional HRP labelling. After washing and transfer to fresh containers, the strips were incubated for 5 min. in 4-chloronaphthol (0.5ng/ml in 50mM Tris/HCl pH 7.4 containing 0.01% H_2O_2), followed by washing in distilled water to stop the reaction.

Results of dot blotting with WAPM as immobilized antigen:

All the antisera were immunoreactive to WAPM (Table 4). Order of immunoreactivity was WAPM = 2 > 1, 5 = 7 > 8 > 3, 4, 6. Thus, of the eight individual antisera, anti-antigen 2 sera displayed the strongest response.

Table 4

Antigen

Antiserum to WAPM	:	+ve to 62.5ng
Antiserum to Ag 1	:	+ve to 62.5ng (< anti-WAPM)
" " " " 2	:	+ve to 62.5ng (= anti-WAPM)
" " " " 3	:	+ve to 125ng
" " " " 4	:	+ve to 125ng
" " " " 5	:	+ve to 62.5ng (< anti-2)
" " " " 6	:	+ve to 125ng (< anti-4 and anti-3)
" " " " 7	:	+ve to 62.5ng (= anti-5)
" " " " 8	:	+ve to 62.5ng (< anti-5)

NRS : negligible

Protein and immunoblotting of specific antigens

Slices of antigen excised from preparative gel bands were run down analytical gels, prepared as described in section 4, except that a larger well former was used to accommodate the gel slices in the stacking gel. The gel slices were soaked in the well. Following electrophoresis, the gels were cut off at the bromophenol blue front and blotted onto PVDF "Immobilon" membrane. The transferred antigens were then re-run on PAGE, blotted and probed with their respective antisera, to give results as shown below in Table 5.

Table 5

Antigen number	a) Rabbit anti-whole adipocyte plasma membrane serum	b) Rabbit anti-specific antigen serum	c) Non-reactive serum
1	not tested		
2	highly reactive	reactive	negative
3	highly reactive	weakly reactive	negative
4	not tested		
5	negative	reactive	negative
6	negative	weakly reactive	negative
7	highly reactive	weakly reactive	negative
8	negative	weakly reactive	negative

Section 13

Establishment of in vivo backfat test in sheep

The Rapid Testing of Antisera for Cytotoxicity to Adipocytes in vivo

Assessment of the respective antisera for their cytotoxicity towards intact adipocytes was required to screen the antisera raised against the 8 specific antigens. The test that was evolved, involved the measurement of the very obvious lesion that develops around the site of injection of a cytotoxic antiserum. These lesions were first observed when sheep were passively immunised subcutaneously with an antiserum raised in horses against whole ovine adipocyte plasma membranes. Briefly, after injection of the antiserum directly into a subcutaneous fat depot, areas of necrotic adipose tissue develop. One week after injection these sites will appear as areas of necrotic fat and inflamed tissue. Later the necrotic tissue degenerates, and after 8 weeks the site appears normal, but is devoid of fat. No such lesion develops around the site of injection of a control antiserum.

Weaned Clun lambs, aged between 10 and 15 weeks were maintained on hay and high energy lamb pellets. A broad band of fleece, from the shoulder to rump and at least 15 cm wide was removed each side of the mid line to expose the skin. Injection sites were then marked with marker pen on the skin. These sites, four per side, were spaced 5 to 10 cm from the mid line and 10 cm apart. Areas known to be relatively devoid of subcutaneous fat were avoided.

The antisera from rabbits immunised with individual ovine adipocyte plasma membrane antigens, positive control antisera from horses immunised with whole sheep adipocytes and negative control antisera from horses and rabbits which had received no immunisation were prepared in an identical fashion, and stored at -20°C. In addition a rabbit anti-whole ovine adipocyte plasma membrane antiserum was prepared to ensure there was no species difference between the antisera of horses (used

initially to prepare antisera) and rabbits. Prior to injection, the antisera were thawed and loaded into individual syringes and warmed to room temperature. A mixture of equal proportions of the individual test antisera was also prepared (excluding the + and - control antisera) for comparison with the antisera from whole membrane.

Each lamb used received one injection of each of the control antisera (+ve horse, -ve horse and -ve rabbit) and the specific antigen antisera mixture. In the remaining four sites, test antisera were allocated randomly. In addition all the antisera were allocated randomly to individual sites on each lamb. Each immunisation was of 1 ml, injected into the subcutaneous fat.

7 days after treatment, lambs were slaughtered by captive bolt, suspended by the hind legs and exanguinated. After all blood flow had ceased, a strip of skin 40 cm wide and including all the injection sites, was carefully peeled back by skinning down from the rump. This was left attached to the carcass at the neck (to avoid confusing the sites). Care was taken to remove the subcutaneous adipose tissue with the skin.

The adipose tissue and skin were examined for any areas of necrosis and damage. Large lesions were obvious, and small lesions were detectable as an area of yellowing tissue. The entire area of skin was examined, and lesions found related to the hide marks on the fleece side of the skin. After measurement with callipers and photography, samples of lesion tissue from each site, and control samples from sites distant from any injection site were removed and stored in formal buffered saline for histology.

The size of the lesions seen in 2 independent studies are shown in Table 6. Photographs are provided as Figures 5 and 6. Figure 5 shows the fat cells in a section from a control animal, the fat cells being relatively well defined. Contrast Figure 6 which is typical of the animals treated according to the invention in which the dark areas represent the damaged regions.

A problem in evaluating the results of Table 6 concerns the effective amounts of antibody raised. The electroelution procedure employed in preparation of the specific antigens has probably resulted in some degradation of proteins. Therefore, the antisera might have been raised against components of the higher relative molecular mass antigens, rather than full length proteins. This makes it particularly difficult to compare amounts of effective epitope, even with the benefit of the dot blotting and ELISA. The high reactivity of antigen 3 in the immunoblotting should be taken into consideration. It can therefore be reckoned that antigens 2, 7 and to a lesser extent 3 were the most effective in these tests, antigen 7 being outstanding. Some effect was seen from antigens 1 and 4.

Table 6

ANTISERUM ADMINISTERED	n	MEAN DIAMETER OF LESION (CM)	COMMENT
Horse anti-ovine adipocyte serum	4	5.0	
Normal (non-immune) horse serum	4	0.0	
Rabbit anti-ovine adipocyte serum	2	2.5	
Normal (non-immune) rabbit serum	2	0.0	
Rabbit anti-antigen 1 serum	2	0.75	
Rabbit anti-antigen 2 serum	2	3.0	(in one experiment only 0.5ml administered)
Rabbit anti-antigen 3 serum	3	0.75	
Rabbit anti-antigen 4 serum	2	1.25	
Rabbit anti-antigen 5 serum	2	0.0	
Rabbit anti-antigen 6 serum	2	0.25	(only 1 of 2 positive)
Rabbit anti-antigen 7 serum	1	7	
Rabbit antiserum mixture 1*	2	4.5	
Rabbit antiserum mixture 2+	2	3.25	

* Mixture 1: Mixture of rabbit anti-antigen, 1, 2, 3, 4, 5 and 6 sera.

+ Mixture 2: Mixture of rabbit anti-antigen, 1, 4, 5, 6, 7 sera.

SEQUENCE LISTING

(1) NUMBER OF SEQUENCES: 1

(2) INFORMATION FOR SEQ ID NO: 1

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 162 amino acids

(B) TYPE: amino acid

(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) FRAGMENT TYPE: N-terminal or internal

iv) SEQUENCE DESCRIPTION: SEQ ID NO: 1

Val Xaa Pro Arg Xaa Xaa Ile

1 5

CLAIMS

1. Antigens present in the plasma membrane of mature ovine white adipocytes, which are not detectable in ovine liver, kidney, spleen, brain, cardiac muscle, skeletal muscle or lung or in ovine erythryocytes, which react with antisera raised against said adipocytes and which on SDS-PAGE gives rise to protein bands of relative molecular mass (r.m.m.) about 101 or about 21 KiloDaltons, respectively, as determined by markers of relative molecular mass 14,300, 21,500, 30,000, 46,000, 69,000, 92,500 and 200,000.

2. An antigen according to claim 1 wherein the r.m.m. is about 21 KiloDaltons and the protein obtained from SDS-PAGE has the partial sequence of amino acids SEQ ID NO: 1:

Val Xaa Pro Arg Xaa Xaa Ile
1 5

3. An antigen according to claim 2 wherein Xaa in position 2 is Ile and in position 5 is Leu or Arg.

4. Antibodies to an antigen claimed in claim 1, 2 or 3.

5. Antibodies according to claim 4 which are monoclonal.

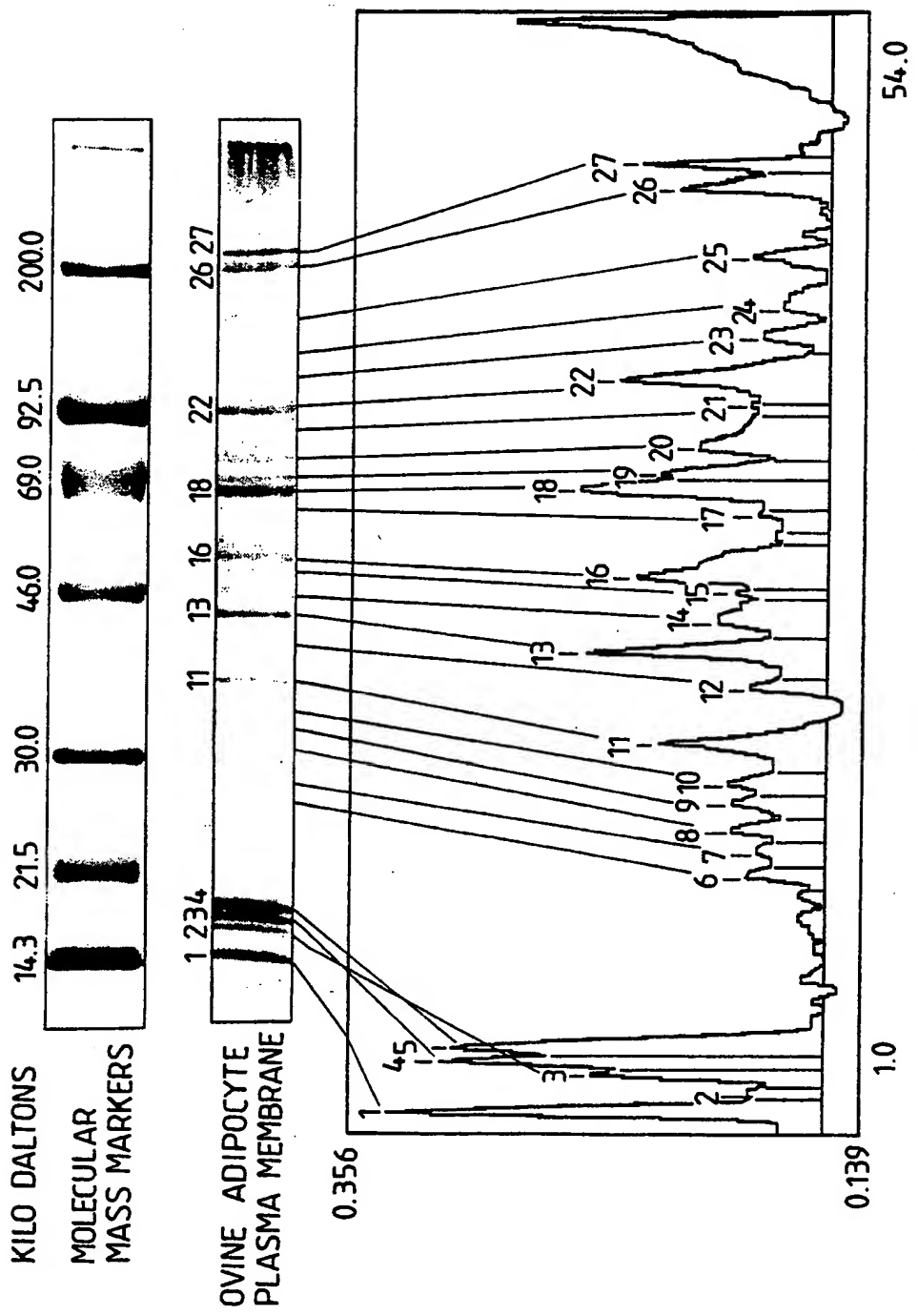
6. A method of reducing fat in sheep by active immunisation which comprises administering to the sheep an amount of an immunogen which comprises an antigen claimed in claim 1, 2 or 3 or a component thereof comprising an epitope and which immunogen has been rendered effective to elicit an immune response to adipocytes.

7. A method according to claim 6 wherein the immunogen is rendered effective to elicit an immune response by conjugation to a carrier protein.

8. A method of reducing fat in sheep by passive immunisation which comprises administering antibodies claimed in claim 4 or 5 to the sheep in an amount effective to immunise the sheep against fat deposition.

9. A conjugate of an antigen claimed in claim 1, 2 or 3 or carrier protein carrying an epitope of a said antigen.

Fig. 1.


KILO DALTONS
MOLECULAR MASS MARKERS
14.3
21.5
30.0
46.0
69.0
92.5
200.0
OVINE ADIPOCYTE PLASMA MEMBRANE
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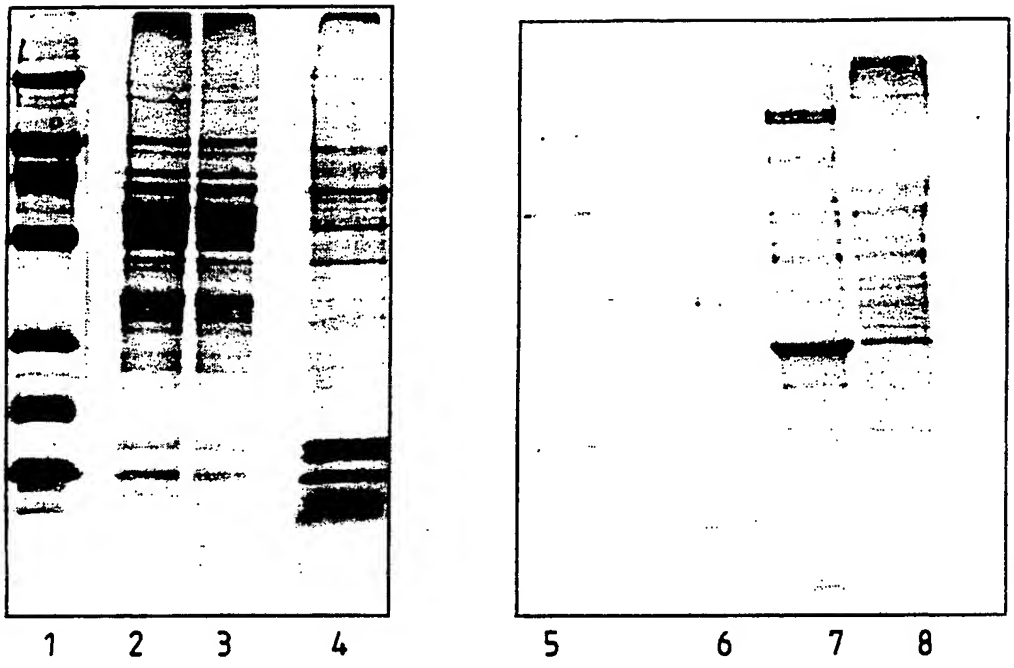
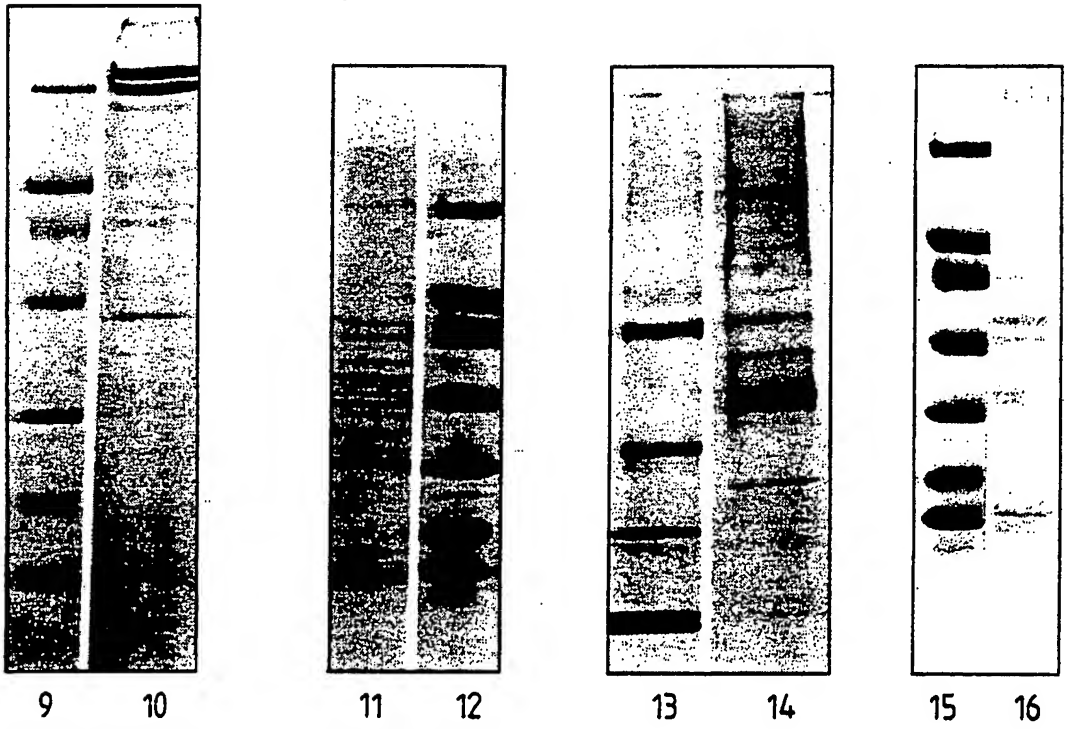
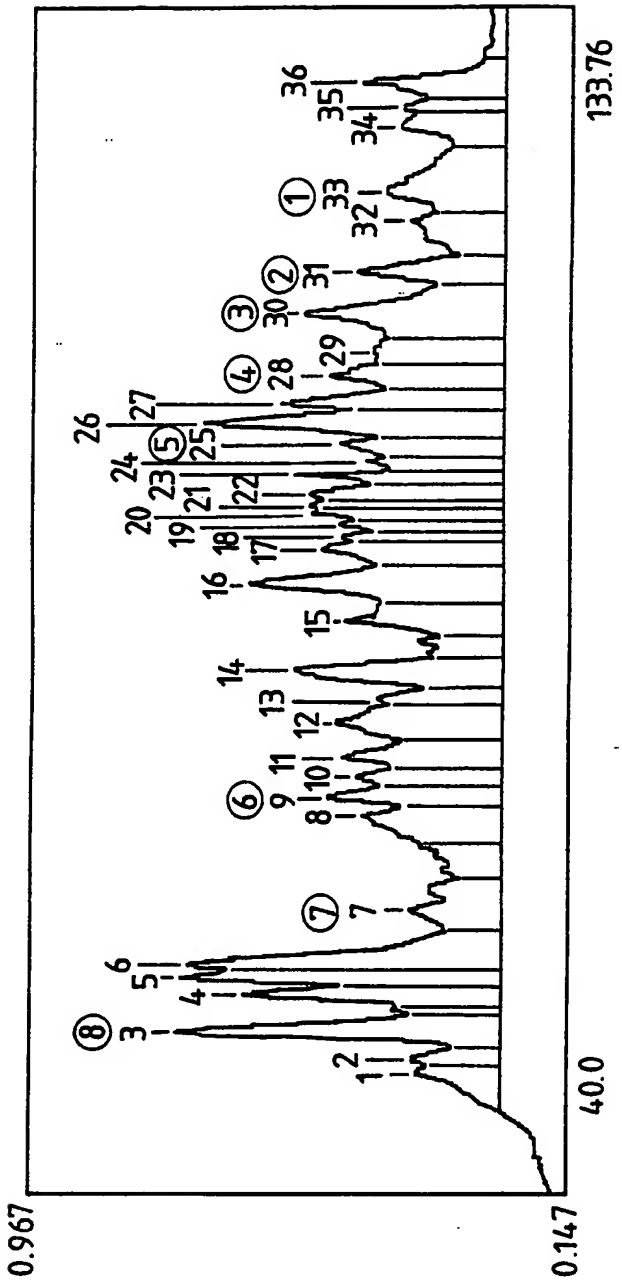
11
13
16
18
22
26 27
0.356
0.139
1.0
54.0

Fig.2.



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0.147
40.0
133.76
1
2
3
4
5
6
7
8
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16
17
18
19
20
21
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⑦
⑧

Fig. 3.

Fig. 4.

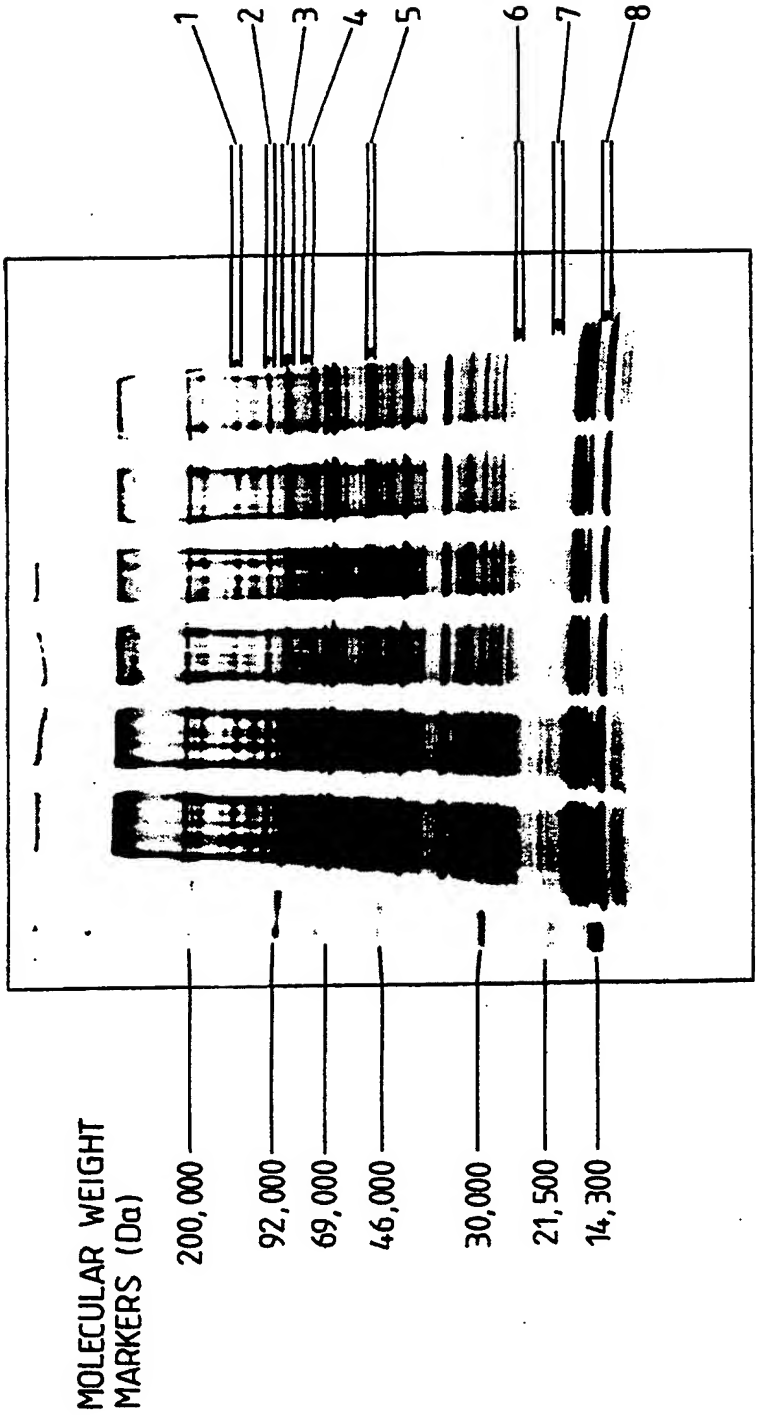
1
2
3
4
5
6
7
8
MOLECULAR WEIGHT MARKERS (Da)
200,000
92,000
69,000
46,000
30,000
21,500
14,300

Fig.5.

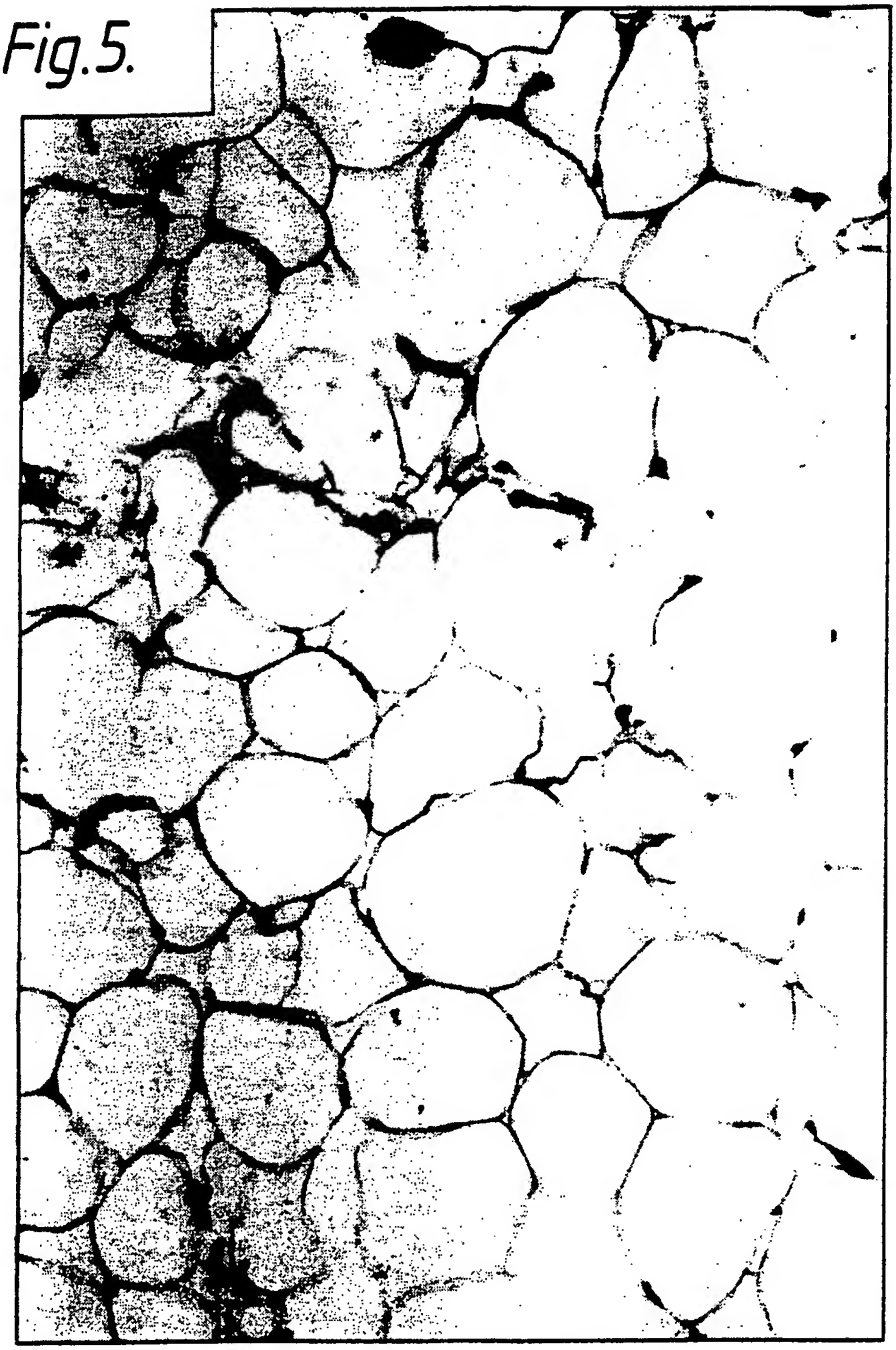

Fig. 6.

INTERNATIONAL SEARCH REPORT

International Application PCT/GB 92/00504

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K15/06; C12P21/08; A61K39/00; A61K39/395

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12P ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
A	COMPARATIVE BIOCHEMISTRY AND PHYSIOLOGY, B : COMPARATIVE BIOCHEMISTRY vol. 98 B, no. 2/3, 1991, OXFORD, GB pages 361 - 367; A.H. NASSAR ET AL.: 'ANTIBODIES TO OVINE ADIPOCYTE PLASMA MEMBRANES RECOGNIZE TISSUE AND SPECIES SPECIFIC PLASMA MEMBRANE COMPONENTS.' cited in the application see the whole document	1-9

-/--

* Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 JUNE 1992	30. 06. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	RYCKEBOSCH A.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	CHEMICAL ABSTRACTS, vol. 112, no. 25, 18 June 1990, Columbus, Ohio, US; abstract no. 233892P, A.I. MOLONEY: 'IMMUNIZING AGAINST ADIPOSE PLASMA MEMBRANES TO REDUCE BODY FAT: EFFECTS ON PLASMA METABOLITES AND INSULIN.' page 495 ; see abstract & BIOCHEM. SOC. TRANS. vol. 18, no. 2, 1990, pages 336 - 337; ---	1-9